PURIFICATION & KINETIC ANALYSIS OF GLUTATHIONE S-TRANSFERASE FROM HUMAN LIVER CELLS

A THESIS

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BY

LESLIE J. BURTON

DEPARTMENT OF BIOLOGICAL SCIENCES

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CDNB	1-chloro-2,4-dinitrobenzene
DCNB	3,4-dichloronitrobenzene
GSH	Reduced glutathione
GST	Glutathione s-transferase
HQ	Hydroquinone
Km	Michaelis constant
K_mGSH	Michaelis constant with GSH
K_m^{CDNB}	Michaelis constant with CDNB
TEMED	Tetramethylethylenediamine
Vm	Maximal velocity
Vmax	Maximal velocity
$Vmax_i$	Maximal velocity in presence of inhibitor

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

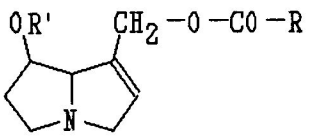
Scientists in the field of chemical carcinogenesis as well as cancer epidemiology have long supported the concept of environmental causation of cancer. Human cancer can result from environmental factors such as cigarette smoking, radiation, industrial pollutants, occupational exposures, pesticides, diet, viruses, pharmaceutical agents, and other life-style factors (Davis, 1989).

Many naturally occurring and anthropogenic chemical carcinogens are ubiquitous in the environment. Miller reported that a high proportion of human cancer is of environmental origin (Miller, 1970). It has also been stated that the chemical carcinogens in our total environment, through lifetime exposure to small amounts of these compounds, may rank as major causes of human cancer (Miller, 1970; Pruessmann, 1976). These small levels of particular carcinogens may actually be "subthreshold" (Pruessmann, 1976). However, this occurrence does not refute environmental carcinogenesis. Cancer in man is multifactorial. Syncarcinogenic activity, and enhancing and modifying factors influencing carcinogenic processes seem to be the central problem of environmental carcinogenesis (Pruessmann, 1976).

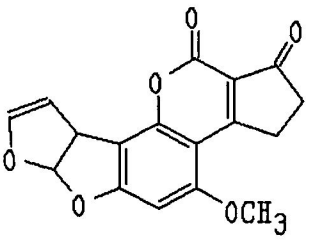
Compounds can influence carcinogenicity in several ways (Davis, 1989). These include: (1) covalent binding of a competing compound with DNA to block or otherwise influence the initiation of carcinogenesis by the toxic compound; (2) trapping or inactivating compounds that deter tumor promotion; (3) biotransforming parent compounds; (4) chemical or physical conversion of metabolites by active catalysis or other physical alteration of carcinogens through food preparation or digestive processes (Davis, 1989). An important aspect of carcinogenesis is that experimentation indicates that the mechanistic action of most environmental carcinogens undergo biotransformation (Miller, 1970). In this respect, compounds such as aflatoxin B_1 and safrole appear to be precarcinogens (Fig. 1) since they are metabolized to carcinogenic and reactive compounds (Miller, 1970).

The biotransformation reactions may be divided into two phases. Phase I of drug/xenobiotic metabolism is concerned with the oxidative and reductive processes involved with the elimination of exogenous/endogenous compounds. The microsomal cytochrome P-450 dependent monooxygenase enzyme oxidation system is instrumental in this respect. This enzyme system requires NADPH in the presence of molecular oxygen to confer its activity, i.e. to oxidize at aliphatic and aromatic carbon, nitrogen and sulfur (Wislocki et al., 1980). However, the cytochrome P-450 oxidation system yields extremely reactive and hence potentially toxic substances such as free

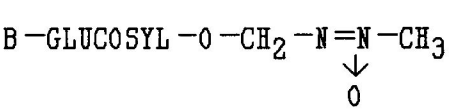
FIGURE 1: Some naturally occuring precarcinogens which are converted in man as well as animals into carcinogenic and reactive structures (Miller, 1970).



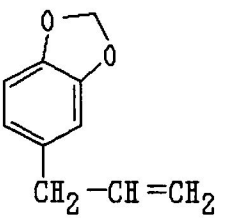
PYRROLIZIDINE ALKALOIDS
(SENECIO, CROTOLARIA AND
HELIOTROPIUM GENERA)



AFLATOXIN B_1
(ASPERGILLUS FLAVUS
STRAIN)



CYCASIN
(CYCAD NUTS)



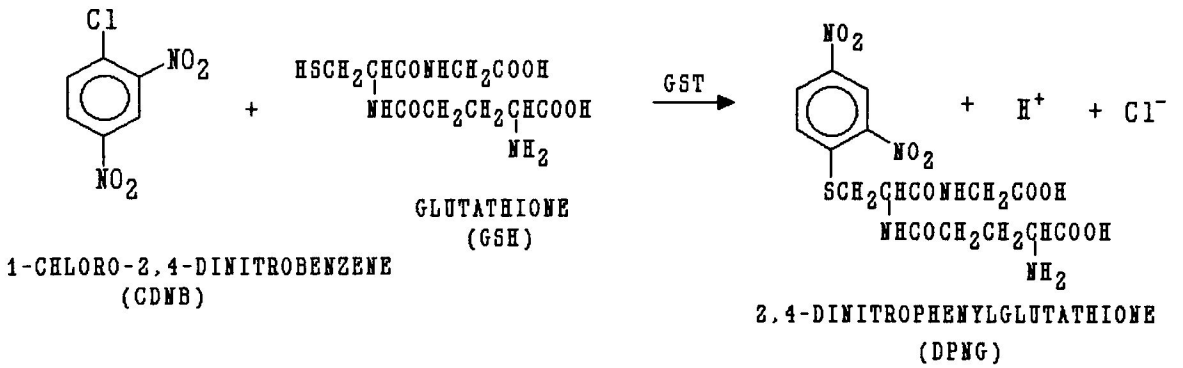
SAFROLE
(OIL OF SASSAFRAS)

radicals, epoxides, and peroxides. (Wislocki et al., 1980). The action of the Phase II enzymes may render these potentially toxic compounds less reactive.

The phase II reactions of drug metabolism involve the conjugation reactions. Glutathione S-transferase, a phase II enzyme, is a major contributor and catalyzes the subsequent conjugation of the toxic compound with glutathione (Wislocki et al., 1980). Its product, GS-X (where GS denotes glutathione and X denotes a potential lipophilic electrophile) is rendered more water soluble and less reactive than the xenobiotic's free form (Fig. 2). Elimination of the foreign compound follows via the bile since S-conjugates are anions (Chasseaud, 1974).

If GST is unable to render these carcinogens inactive, then they may interact with informational macromolecules and ultimately induce malignant cancers. This mechanism (Fig. 3) would generally follow that of a S_N2 reaction in which a relatively positive or electrophilic atom in the carcinogen combines with the relatively negative or nucleophilic atoms of the molecules attacked in cells (Miller, 1970; Miller & Miller, 1971).

FIGURE 2: Catalysis of the conjugation of glutathione to organic electrophiles such as 1-chloro-2,4-dinitobenzene (CDNB) to form thioesters (Adams and Sikakana, 1990).

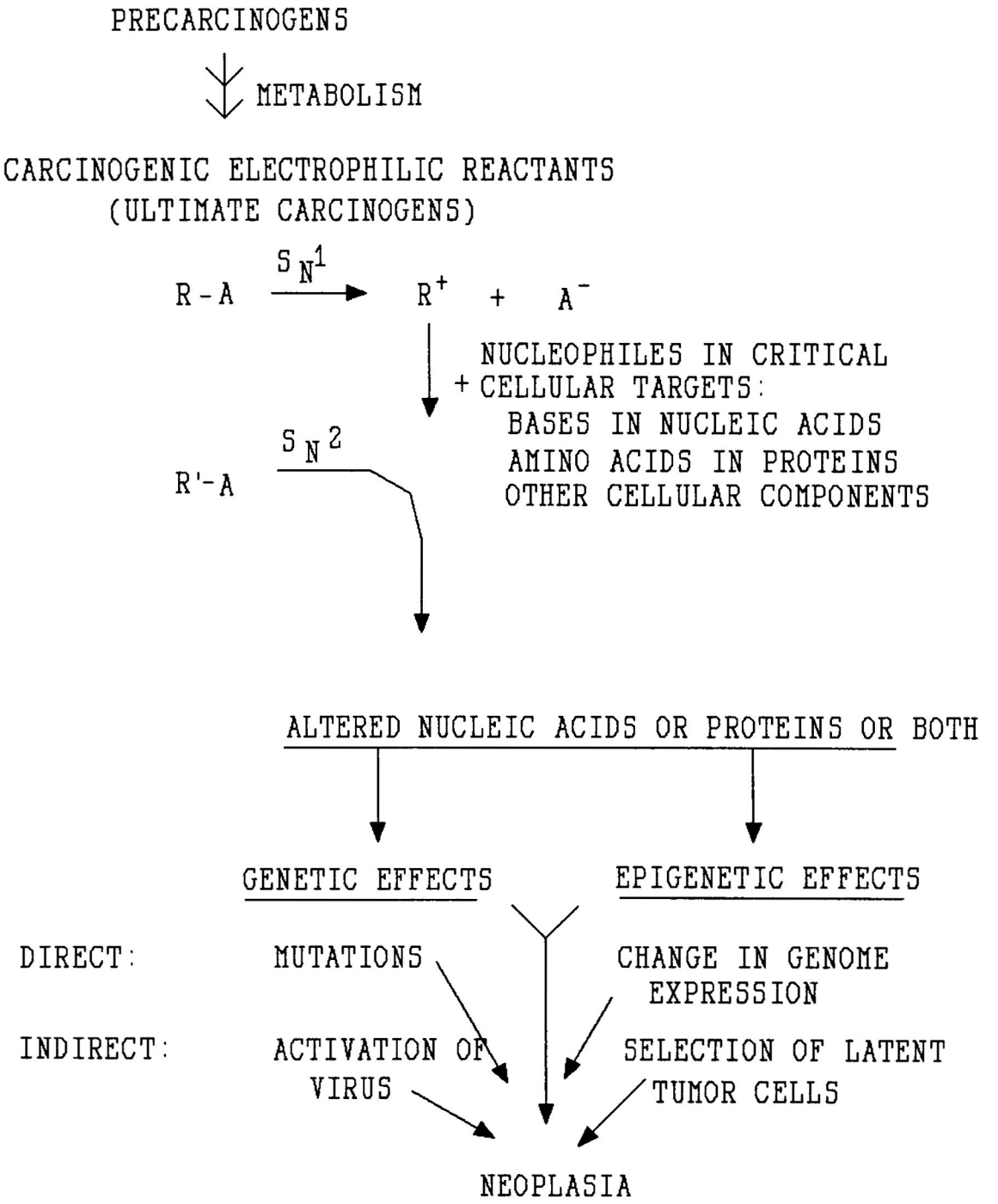


1-CHLORO-2,4-DINITROBENZENE
(CDNB)

GLUTATHIONE
(GSH)

2,4-DINITROPHENYLGLUTATHIONE
(DPNG)

FIGURE 3: Possible mechanisms of carcinogenesis by the ultimate carcinogenic electrophilic reactants derived from chemical carcinogens or precarcinogens (Miller, 1970).


PRECARCINOGENS
METABOLISM
CARCINOGENIC ELECTROPHILIC REACTANTS
(ULTIMATE CARCINOGENS)
R-A
S_N1
R^+ + A^-
+ NUCLEOPHILES IN CRITICAL CELLULAR TARGETS:
BASES IN NUCLEIC ACIDS
AMINO ACIDS IN PROTEINS
OTHER CELLULAR COMPONENTS
R'-A
S_N2
ALTERED NUCLEIC ACIDS OR PROTEINS OR BOTH
GENETIC EFFECTS
EPIGENETIC EFFECTS
DIRECT:
MUTATIONS
CHANGE IN GENOME EXPRESSION
INDIRECT:
ACTIVATION OF VIRUS
SELECTION OF LATENT TUMOR CELLS
NEOPLASIA

Once the malignant cancers are induced, irreversible processes occur during the initiation stage of preneoplasia. Alterations of specific genes, including the activation of proto-oncogenes, occur (Sato, 1989). The outcome is the development of tumor cells possessing elevated glutathione s-transferase activity (Sato, 1989; Hayes et al., 1990; Rao, 1992).

The dilemma arises in attempting to treat these cells. Many chemotherapeutic agents are also inactivated by glutathione (Griffith, 1989). Hence, these carcinostatic compounds may serve as potential substrates of GST and, as such, are rendered ineffective. However, these tumors can be sensitized to the effects of the chemoprotectant by minimizing GST activity (Griffith, 1989).

Rationale

The purpose of this study is to examine the interaction of GST with its substrates via kinetic analysis. The generated results would allow the proposal of a plausible reaction mechanism. As such, the form of enzyme that would best interact with a putative chemotherapeutic agent can be determined. Hence, the chemoprotectant can be chemically designed such that its therapeutic efficacy is maximal.

CHAPTER II

LITERATURE REVIEW

As reported by Meister (1980), in 1879 Baumann, Preusse and Jaffe demonstrated that the administration of bromobenzene and chlorobenzene to canines resulted in urinary excretion of compounds referred to as mercapturic acids. S-substituted derivatives of N-acetylcysteine are referred to as mercapturic acids (Boyland and Chasseaud, 1969). The source of the cysteine residue was thought to have derived from dietary proteins. However, in 1937, Stekol refuted this idea upon supplementing the food of animals with glutathione (reported by Boyland and Chasseaud, 1969). There was no net increase in mercapturic acid formation resulting from the diet or from depletion of cysteine from tissue proteins. Several mechanisms of mercapturic acid formation were proposed (Stekol, 1939; Smith et al., 1950; Mills and Wood, 1956; Barnes et al., 1959; Bray et al., 1959; Waelsch, 1930; Brand and Harris, 1933). It was Booth and his colleagues in 1961, who isolated a protein/enzyme that catalyzed conjugation of glutathione with foreign compounds, including some that were metabolized to mercapturic acids. Grover and Sims (1964) referred to this enzyme as glutathione S-aryltransferase because of its apparent specificity for glutathione and

compounds possessing an aromatic ring. Several other enzymes capable of catalyzing glutathione conjugation were later discovered and purified. These enzymes are collectively known as glutathione S-transferases.

Glutathione S-transferases (GSTs) are comprised of (1) glutathione S-aryltransferase, (2) glutathione S-epoxidetransferase, (3) glutathione S-alkyltransferase, (4) glutathione S-aralkyltransferase, and (5) glutathione S-alkenetransferase (Boyland and Chasseaud, 1969). However, Jakoby (1980) contend that the term "Glutathione Transferases" is a misnomer since these enzymes should not be viewed as participating in only the transfer of glutathione, but rather as proteins that catalyze any reaction in which glutathione thiolate anion participates. GSTs catalyze the conjugation of GSH to xenobiotics and other electrophilic compounds possessing aromatic and other cyclic rings that have labile nitrogen or halogen groups. These proteins may exist in soluble and membrane-bound forms. Glutathione S-transferases collectively represent a family of dimeric and trimeric enzymes which have been isolated in the cytosol and microsome, respectively (Hayes et al., 1991; Adams and Sikakana, 1990). The membrane-bound microsomal GST, which is less active than cytosolic GST, represents only one type while all the other types can be found in the cytosol (Meikle, et al., 1991). Hence, only the cytosolic form will be considered. These enzymes may be found in various species, from Escherichia coli

to humans, and may exist as homodimers or heterodimers (Pickett, 1989; Adams and Sikakana, 1990; Ivanetich and Goold, 1989). Each subunit is found to have a molecular weight ranging from 17kd to 26kd and is characterized on the basis of its isoelectric points (Hayes et al., 1991; Habig and Jakoby, 1981). Approximately eleven subunits have been identified in the rat and, in the nomenclature used, each subunit is given a number in the chronological order of its characterization (Jakoby et al., 1984). The complete amino acid sequences have been deduced for subunits 1 (Pickett et al., 1984; Lai et al., 1984), 2 (Telakowski-Hopkins et al., 1985), 3 (Ding et al., 1985), 4 (Lai et al., 1988), 6 (Abramowitz and Listowsky, 1987) and 7 (Suguoka et al., 1985). Mannervik et al., (1985) and Mannervik (1985) report that the sequences, based on structural data (Table 1), suggest that the subunits fall into three categories or classes: the alpha class (subunits 1 and 2), the mu class (subunits 3, 4, and 6), and the pi class (subunit 7).

The mode of action of these enzymes are presently unclear. Unlike other prominent conjugation processes, GSH conjugation does not require utilization of ATP (Chasseaud, 1979). Al-Kassab et al., (1963) suggested that rat GST provided a "charged" GSH for conjugation with an electrophile. The rate of the non-enzymatic reaction of the electrophilic

TABLE 1: Similarities of gutathione s-transferases

Transferase (pI)	Amino-terminal amino acid sequence
Class alpha* (pI>8)	
Rat 1-1 (cDNA)	*M*SGKPVLHYFNARGRMECIRWLLAAA
Rat 1-2	PGKPVLHYFNGRGRMEPI
Rat 2-2	PGKPVL*H*YF
Rat 2-2 (cDNA)	*M*PGKPVLHYFDGRGRMEPI
Class mu (pI 6.5)	
Human μ	PMILGYWDIRGLAHAIRLLLEYT
Human γ (pI 5.5)	not determined
Rat 3-3	PMILGYWNVRGLTHPIRLL
Rat 4-4	PMTLGYWDIRGLAHAIRLFLEYTDT
Mouse MIII	PMILGYWNVRGLTHPIRMLLQYT
Class pi (pI 4.8)	
Human π	PPYTVVYFPVRGRCAALRMLLAD
Rat 7-7	PPY*T*IVYFPV
Mouse MII	PPYTIV YFPVVDGCEAM
Microsome (pI ?)	Lacks homology with any of the cytosolic isozymes

*Class alpha also comprises human transferases α-ϵ and mouse transferase MI. These proteins are amino-terminally blocked and are not listed owing to lack of sequence information.

Italicized residues indicate tentative assignments or initiator methionine.

substrate with GSH increases proportionately to the increase of ionized GSH (Chasseaud, 1979). This outcome, therefore, suggests that GS^- is the reacting nucleophile. Keen et al., (1976) reported that human (alpha class; see Table 1) β and δ GSTs promote ionization of the sulfhydryl group of GSH by lowering its pK (pK = 9.2) and thereby increasing its nucleophilicity. In addition, GST would bind the electrophile promoting preferential interaction with the GS^- anion. Reduction of the pK of the GSH thiol is thought to be a major contributor to GST's mode of action. Awasthi et al., (1987) contend that a single histidine residue is responsible for this reduction since substitution of a single histidine residue with a basic group resulted in the loss of human ψ (Table 1) GST activity. It has also been suggested that an arginine residue is associated with GSH binding and anion recognition (Schasteen et al., 1983). On the other hand, Kong et al., (1992) reported that a tyrosine residue on human π GST is responsible for GSH ionization.

Characterization of the kinetic mechanism of GST is equally perplexing. Investigators have reported that studies on YaYa GST from male Sprague-Dawley rats suggest that the kinetic mechanism of substrate addition is likely to be random (Schramm et al., 1984). In addition, competitive inhibition studies reveal a single binding site for a product analog. This occurrence is consistent with the presence of a single catalytic site (Schramm et al., 1984). Danielson and

Mannervik (1988) reported that GST from rat liver exhibits paradoxical inhibition which may be explained by substrate-inhibitor-enzyme complexes in a random-order sequential mechanism. However, experimental support for kinetically significant conformational changes under steady-state conditions is lacking. Ivanetich and Goold (1989) also endorsed the rapid random equilibrium sequential Bi Bi mechanism for human placental glutathione S-transferase (Ivanetich and Goold, 1989). In addition, these investigators suggested that the kinetic mechanism of the GST is isoenzyme-dependent. Some isoenzymes (for example rat's YaYa GST) appear to follow conventional hyperbolic kinetics while others (for example rat's 2-2, 3-3, 3-4 GSTs) exhibit non-hyperbolic kinetics (Schramm et al., 1984; Ivanetich et al., 1990). The steady-state random sequential Bi Bi mechanism may be sufficient to explain this phenomenon in rat hepatic GST isoenzymes (Ivanetich et al., 1990). Phillips and Mantle (1991) reported that by using a computer simulation program (EKPLOT) a model was developed that suggested the presence of an allosteric site on mouse GST YfYf. Perhaps this allosterism accounts for the deviation from hyperbolic kinetics.

There appears to be little work reported on human hepatic glutathione s-transferases. In some respects this is surprising since these forms of GST have received considerable interest as possible early markers for hepatocellular toxicity

and carcinogenicity (Beckett et al., 1985a; Beckett et al., 1989a; Beckett et al., 1989b; Hayes et al., 1990; Hussey et al., 1986; Hayes et al., 1988; Sherman et al., 1983; Beckett et al., 1987; Sherman et al., 1983; Beckett et al., 1985b; Gow et al., 1987). Hence, the aims of this study are to purify glutathione s-transferase from human hepatoma cells and to elucidate the kinetic parameters of human hepatic GST. The data generated may be used to derive a possible reaction mechanism.

CHAPTER III

MATERIALS AND METHODS

Cell Culture

Untreated human hepatoma cells (cell line HEP-G2) were purchased from the American Type Culture Collection. The HEP-G2 cells were incubated (in 5% CO_2) in sterile 75cm^2 flasks at 37^{o}C in Dulbecco Modified Eagle's Medium with 4.5g/L glucose supplemented with 10% Fetal Bovine Serum and an Antibiotic-Antimycotic mix (penicillin, streptomycin and amphotericin B). The cells were kept in log phase by weekly subculture.

Subculture

Subculturing entailed decanting the medium and washing the cells twice with 5 ml Hanks Balanced Salt Solution. The salt solution was decanted and the cells incubated with 2.5 ml Trypsin/EDTA for 2-5 min. for cell detachment. The cells were then transferred to a sterile culture tube and centrifuged at approximately 1000 xg for 4 min. The supernatant was decanted and the pelleted cells were resuspended in 2 ml Dulbecco Modified Eagle's Medium. The cells were aliquoted, 0.2 ml per 10 ml medium, into culture flasks.

Harvest

The medium was decanted by aspiration. The cells were washed twice with 5 ml (4°C) saline. The saline solution was decanted and 0.5 ml of 0.01 M potassium phosphate buffer with 0.1% KCl (pH 7.3) was added to the flask. The cells were detached from the flasks' surface with a rubber policeman and homogenized using a motor driven pestle. The homogenate was centrifuged at 10,000 xg in a Beckman J2-21 centrifuge at 4°C for 30 minutes. The supernatant was decanted and stored at -70°C until needed.

Purification of GST from HEP-G2 Cells

HEP-G2 cells were grown in culture to provide a source of GST. The cells were harvested and homogenized on ice at 4°C. The homogenate was concentrated to a volume of 1 ml via an Amicon ultrafiltration unit. The 1 ml sample was layered on a glutathione-agarose affinity column and eluted with three buffers: (A) 10 mM Tris HCl, pH 8, (B) 10 mM Tris HCl, pH 8, with 5mM GSH, (D) 10 mM Tris HCl, pH 8, with 0.1 M KCl. The eluent containing 0.1 M KCl was dialyzed against buffer A using the Spectra/Pore molecular porus membrane with a molecular weight cut-off of 6000-8000 daltons. The eluents and dialysate were then concentrated to 5 ml via ultrafiltration. The protein content of the samples were determined by means of BioRad's microprotein assay.

Protein Assay

Briefly, 0.8 ml of standards (known quantities of Bovine Serum Albumin) and appropriately diluted samples were placed in clean, dry test tubes. The "blank" consisted of 0.8 ml sample buffer. The contents of each test tube was vortexed, avoiding excess foaming, following the addition of 0.2 ml BioRad's dye reagent concentrate. After an incubation period of 15 min. at room temperature, the OD_{595} versus the reagent blank was determined using the Beckman DU-8 Spectrophotometer. A standard plot of OD_{595} versus concentrations of standards was generated from which the concentrations of the unknown samples was determined.

Assessment of Kinetic Mechanism

A GST enzyme assay was executed according to Corrigall et al., (1989). The reaction mixture contained 3.33 mM potassium phosphate with 0.1% KCl (pH 7.2), 0.5 ug GST, and the primary (reduced glutathione; GSH) and secondary (1-chloro,2,4-dinitrobenzene; CDNB) substrates. One substrate was varied between 1.00 mM - 2.00 mM while the other was held at a constant concentration while maintaining the final volume of the reaction mixture at 3 ml. This series of experiments were repeated by alternating the constant and variable substrates.

The optical density was determined at 340 nm (absorbance maximum of the product) with a Beckman DU-64 Spectrophotometer equipped with a kinetics compuset module. This spectrophotomer has the capacity to take absorbance measurements at time intervals as small as 3 seconds. The initial rates of reaction were monitored in 12 seconds at 25°C.

Determination of the Composition of GST

SDS-PAGE was employed to assess GST subunits according to the method of Laemmli (1970). Fifteen milliliters (15 ml) of deionized 30% acrylamide:bisacrylamide (29:1) was mixed with 15 ml of 1.5 M Tris base (pH 8.8 with HCl) containing 0.4% (v/v) SDS and 100uL of ammonium persulfate (100 mg/ml made on the day of use). The volume was brought to 60 ml with deionized water. This yielded a 7.5% gel. Thirty microliters (30 uL) N,N,N^1,N^1-Tetramethylethylenediamine (TEMED) were added to catalyze polymerization. Bio-Rad's Protean II xi gel electrophoresis cell was utilized and the manufacturer's instructions were followed for assembly. The 60 ml unpolymerized gel mix was poured into the prepared plates and gently layered with a few milliliters of saturated butanol/H_2O to form a flat surface while the acrylamide polymerized. After one hour, the butanol/H_2O was decanted, and a top "stacking" gel [1 ml 30% acrylamide/bisacrylamide, 4 ml H_2O,

5 ml 0.25 M Tris, pH 6.8, 0.2% (v/v) SDS, 100 uL ammonium persulfate, 7 uL TEMED] was poured to fill the upper 3 cm of the gel. The well-forming comb was inserted and the stacking gel was allowed to polymerize. The gel was attached to the electrophoresis unit and approximately 1.5 L 1X electrode buffer [0.025 M Tris base with 14.4 g glycine, 0.1% (v/v) SDS] was added to the bottom chamber and 500 ml was added to the top chamber. Ten microliters (10 uL) samples (1-100 ug protein) were mixed with 10 uL of 2X sample buffer [0.125 M Tris, pH 6.8, 4% (v/v) SDS, 10% glycerol, 0.02% (w/v) bromophenol blue, 5% (v/v) B-mercaptoethanol]. The mixture was then heated to 95^{o}C for 3 min. and loaded on the gel. The molecular weight standards, which were treated identically as the sample, were then loaded on the gel. Electrophoresis began at a constant 100 volts and continued until the dye front approached the terminus of the gel. After electrophoresis, the gel was removed from the unit and stained for at least 30 minutes with 0.1% Coomassie Blue in fixative (40% methanol, 10% acetic acid). The gel was then destained with 40% methanol:10% acetic acid.

CHAPTER IV

RESULTS

Glutathione S-transferase was purified from HEP-G2 human hepatoma cells by affinity chromatography on cross-linked 4% beaded epoxy-activated agarose with insolubulized GSH. The 9,000 x g supernatant extract from these cells was applied to a 20 cm x 1.5 cm affinity column, which was eluted step-wise with three buffers (Buffers A, B, and D). Figure 4 depicts a typical elution profile.

Elution of the column with buffers B and D resulted in the detection of two putative forms of GST, identified as low-affinity (fraction B) and high-affinity forms (fraction D). This designation was given to the peaks because of the relative ionic strength of the buffers needed to elute each form.

During a typical purification protocol, fraction A represented 84% of the soluble cytosolic protein placed on the column while fractions B and D represented 0.64% and 0.35%, respectively (Table 2). Thus, the GST isoenzymes comprised less than one percent of the total recovered cytosolic proteins.

FIGURE 4: Elution profile of glutathione s-transferase. HEP-G2 supernatant was loaded on a glutathione-agarose affinity column. The column was eluted successively with buffers A, B, and D.

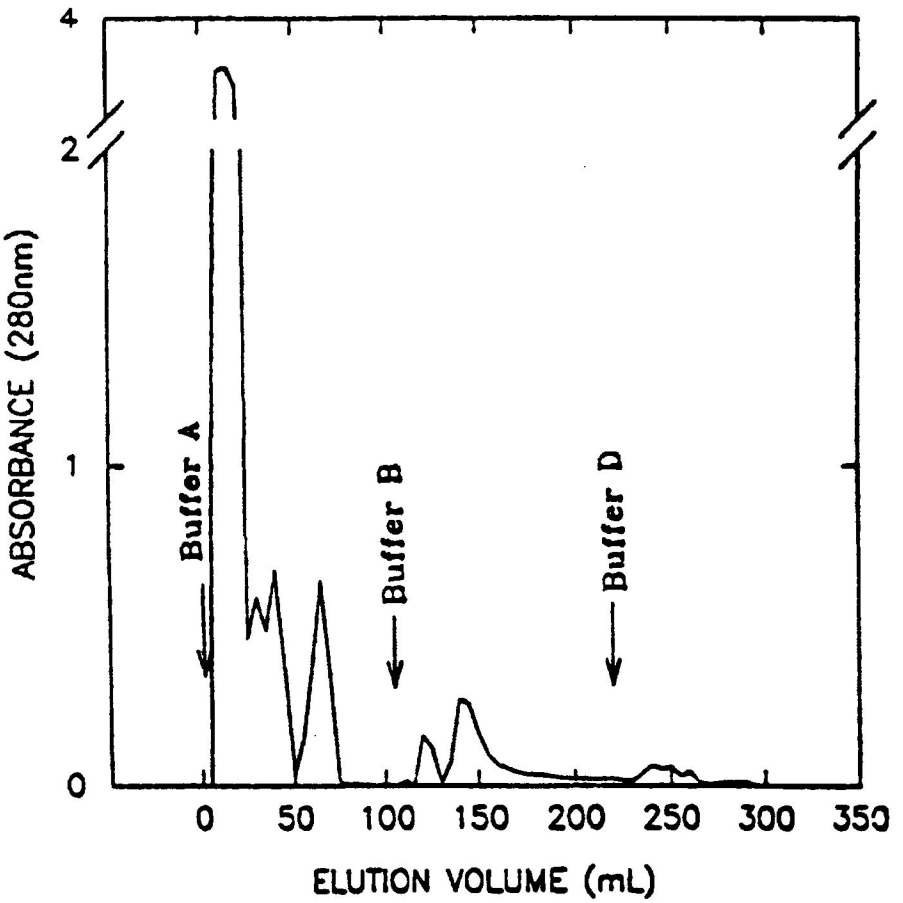

4
2
1
0
ABSORBANCE (280nm)
Buffer A
Buffer B
Buffer D
0
50
100
150
200
250
300
350
ELUTION VOLUME (mL)

TABLE 2: Glutathione s-transferase purification.

SAMPLE	SPECIFIC ACT. (nmol/min/mg protein)	FOLD PURIFICATION	% YIELD*
HEP-G2 Supernatant (9,000 x g spin)	1742.62	-	-
Fraction A	514.62	-	84.0
Fraction B	62,907.42	36.10	0.64
Fraction D	162,769.64	93.41	0.35

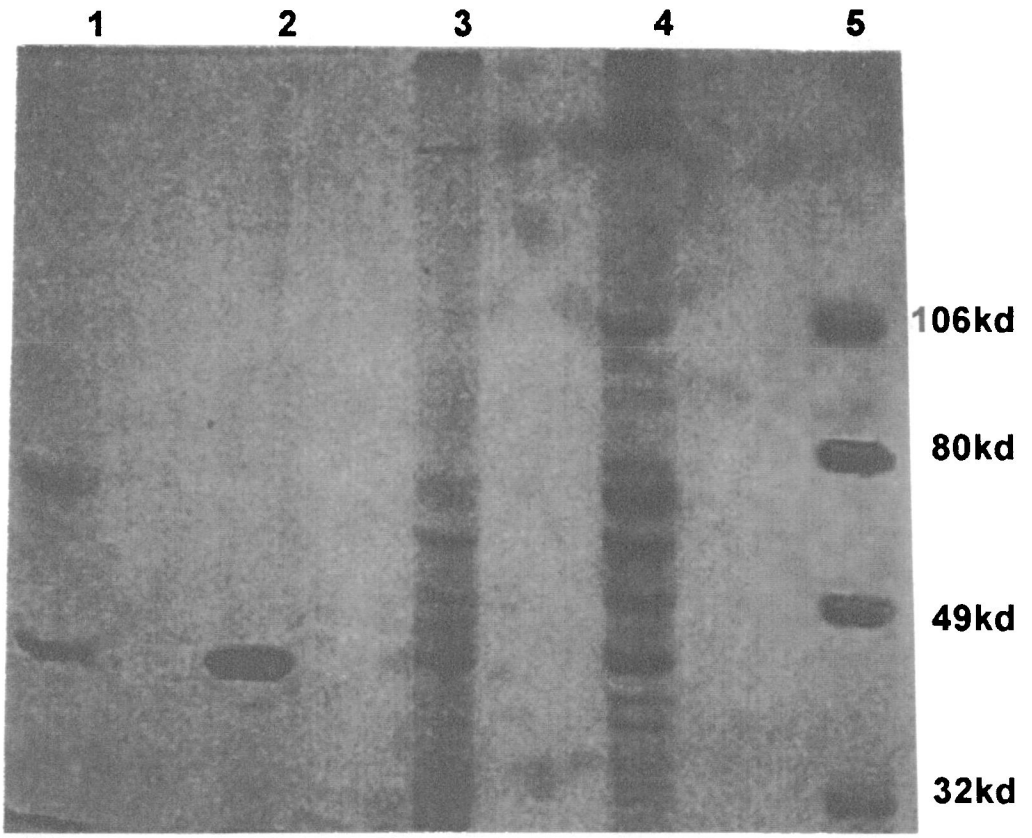
*% yield of total protein from the 9,000 x g supernatant was determined by successive protein assays at each purification step.

Both fractions B and D exhibited GST activity with the substrates CDNB and GSH (Table 2) while specific activity with DCNB and GSH was negligible (data not shown). Since fraction D possessed the greatest amount of GST activity it was used as the source of enzyme in subsequent studies.

Electrophoretic Analysis of Purified GST

The electrophoretic mobility of glutathione s-transferase was examined and the molecular weight of both forms of GST was determined via SDS-PAGE as described by Laemmli (1970). The stacking gel and the separating gel concentrations were 3% and 7.5%, respectively. Phosphorylase b (106,000 daltons), Bovine Serum Albumin (80,000 daltons), Ovalbumin (49,500 daltons) and Carbonic anhydrase (32,500 daltons) were used as standards for characterization of molecular size. The low-affinity isoenzyme studied yielded a single band of 47,969 daltons as compared to the protein markers. The high-affinity isoenzyme studied yielded a band of 45,115 daltons in addition to a second band of 75,218 daltons as compared to the protein markers (Fig. 5).

Figure 5: Sodium Dodecyl Sulfate Polyacrylamide Gel. The electrophoretic mobility of the GST isoenzymes were analysed via SDS-PAGE. The gel consists of 7.5% separating gel and 3% stacking. Lane 1 displays the high affinity GST isoenzymes (fraction D); lane 2 displays the low affinity GST isoenzymes (fraction B); lane 3 displays fraction A; lane 4 displays the 9,000 x g supernatant and lane 5 displays the protein markers.


1
2
3
4
5
106kd
80kd
49kd
32kd
D
B
A
9,000 x g supnt.
MW markers

Determination of Optimal Assay Conditions

In order to determine the optimal conditions under which the enzyme would exhibit maximal activity, the substrate concentrations and pH of the reaction mixture (3.33 mM potassium phosphate with .1% KCl, 1 mM GSH, 5 mM CDNB, GST, dH_2O to final volume of 3 ml) were varied. The pH profile (Fig. 6) revealed that enzyme activity is maximal at pH 7.2.

The CDNB profile (Fig. 7) reveals that activity is maximal at a 5 mM concentration. Linearity was lost above concentrations of 5 mM CDNB. However, at physiological pH (7.2) and 5 mM CDNB, the reaction appeared to remain linear from 1 mM GSH - 3 mM GSH (Fig. 8).

Another study involved varying the amount of GST in the assay. Figure 9 shows that the amount of activity increased with increasing amounts of GST and plateaued at approximately 1.4 ug protein. The optimal conditions for kinetic analysis were hence defined as 5 mM CDNB and 3 mM GSH representing saturating substrate levels, pH 7.2, and 0.5 ug GST. These conditions were maintained throughout all kinetic assays.

FIGURE 6: Glutathione s-transferase activity with varying pH. Specific activity is measured over a pH range of 6.2-7.4.

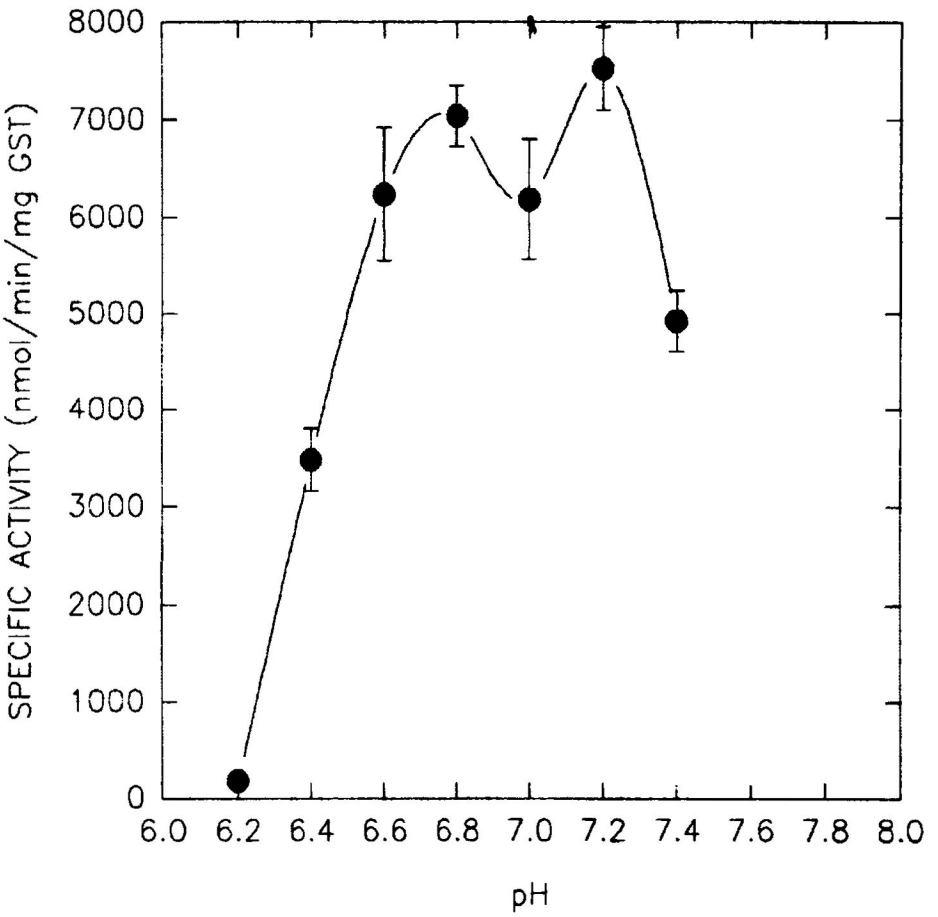

SPECIFIC ACTIVITY (nmol/min/mg GST)
8000
7000
6000
5000
4000
3000
2000
1000
0
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
pH

FIGURE 7: Glutathione s-transferase activity with varying CDNB concentrations. Activity was measured at pH 7.2 and 1 mM GSH.

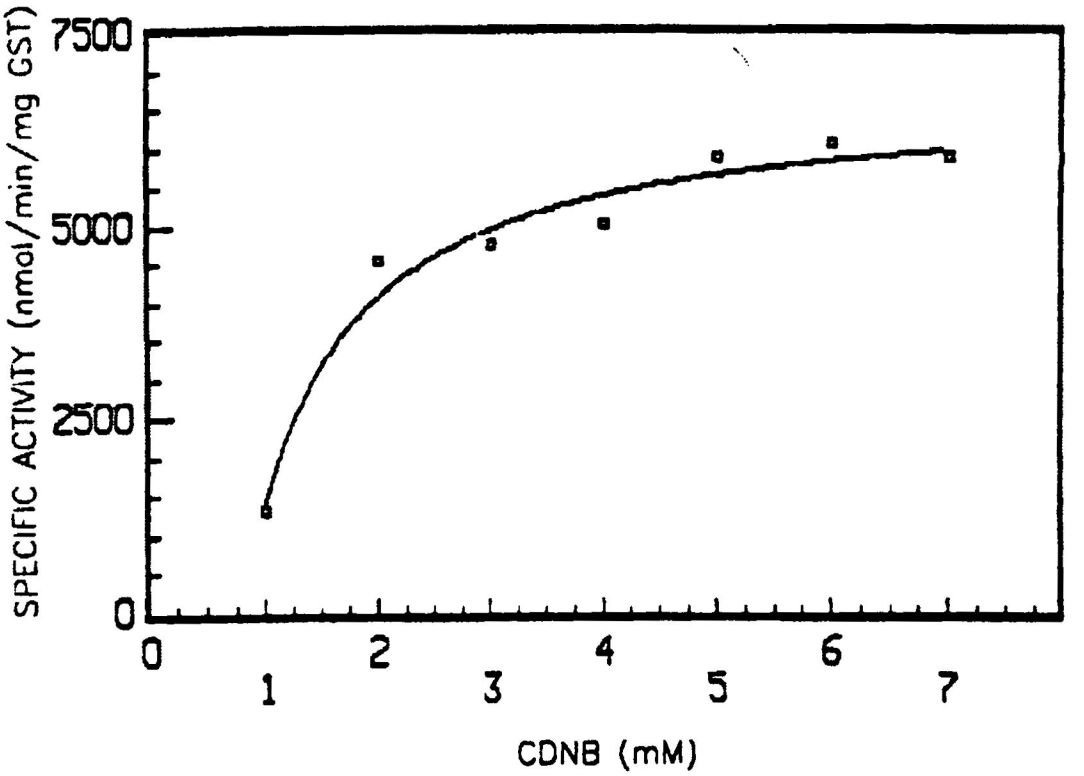

SPECIFIC ACTIVITY (nmol/min/mg GST)
7500
5000
2500
0
0
1
2
3
4
5
6
7
CDNB (mM)

FIGURE 8: Glutathione s-Transferase activity with varying GSH concentrations.

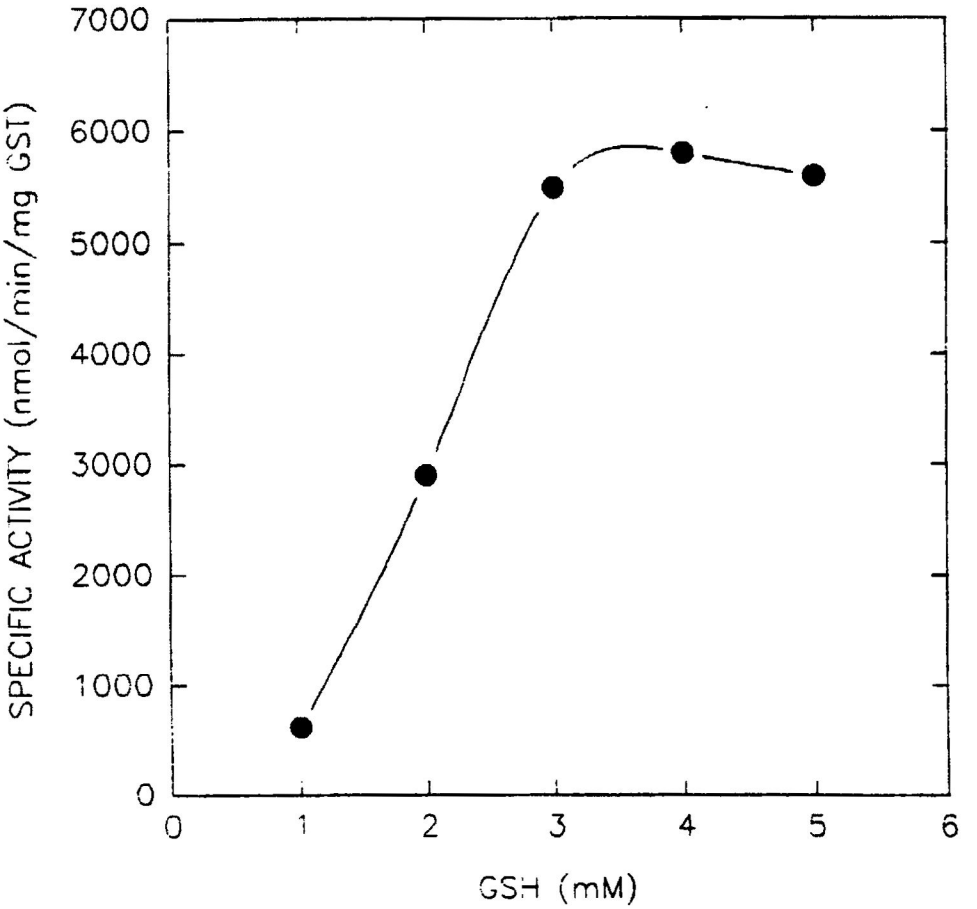
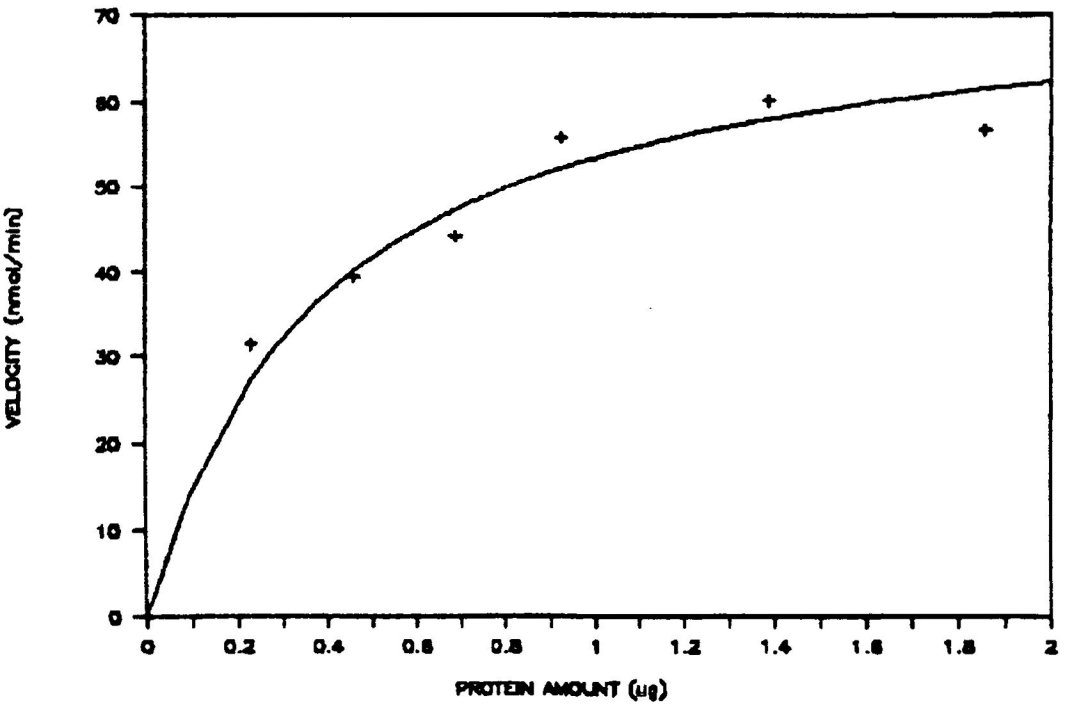

7000
6000
5000
4000
3000
2000
1000
0
SPECIFIC ACTIVITY (nmol/min/mg GST)
0
1
2
3
4
5
6
GSH (mM)

FIGURE 9: Glutathione s-transferase activity with varying GST (fraction D) amounts. Fraction D was assayed following dialysis to remove excess salt.


VELOCITY (nmol/min)
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
PROTEIN AMOUNT (µg)

Kinetic Analysis

The initial rate kinetics with varying CDNB gave a pattern of intersecting lines which met near the abscissa of the double reciprocal plot (Fig. 11). Analysis of the experimental data in terms of the initial rates gave a Michaelis constant (K_m) value of 2.502 mM ± 0.003 mM in the absence of the inhibitor (Figs. 10 and 11). The apparent Km value for varying GSH is 1.611 mM ± 0.548 mM in the absence of the inhibitor (Figs. 12 and 13). The maximal rate of catalysis was 0.188 nmol/min ± 0.032 nmol/min for varying GSH and 0.042 nmol/min ± 0.025 nmol/min for varying CDNB in the absence of the inhibitor (Figs. 11 and 13). Inhibition studies were also conducted. Initial rates were recorded in the presence of the inhibitor, 0.6 mM hydroquinone (HQ) also known as 1,4-benzenediol. In separate experiments, incubation of hydroquinone in the absence and presence of enzyme did not result in the loss of hydroquinone. Hence, there is no enzymatic or non-enzymatic hydrolysis of hydroquinone. The Michaelis constant, in the presence of HQ, for varying GSH was 0.391 nmol/min ± 0.042 nmol/min (Figs. 12 and 13). The apparent K_m value for varying CDNB in the presence of hydroquinone was 2.514 nmol/min ± 0.124 nmol/min (Figs. 10 and 11). The maximal catalytic rates, while varying CDNB and GSH in the presence of hydroquinone, were 0.013 nmol/min ± 0.005 nmol/min and 0.083 nmol/min ± 0.002 nmol/min, respectively (Figs. 11 and 13; refer to Table 3 for kinetic parameters).

FIGURE 10: Hyperbolic plot of GST (D) initial velocity v/s varying CDNB concentrations, with 3 mM GSH remaining constant, in the presence (closed circles) and absence (open circles) of the inhibitor, hydroquinone.

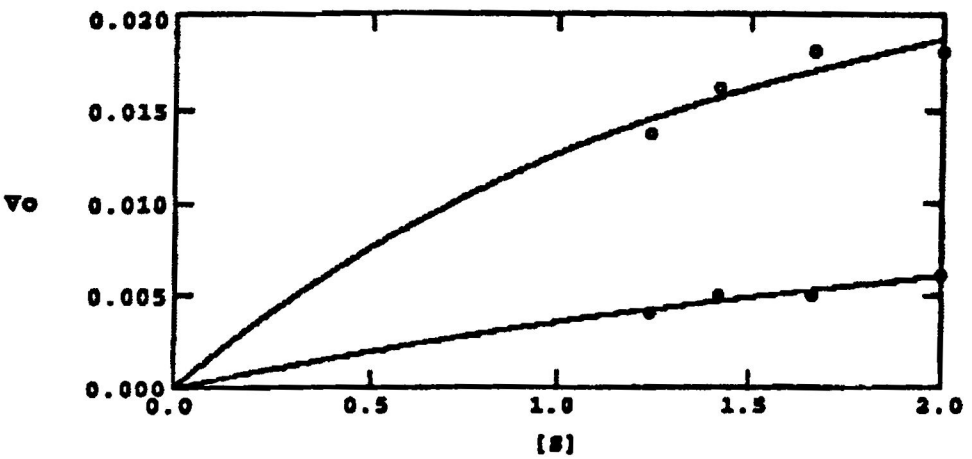

0.020
0.015
Vo
0.010
0.005
0.000
0.0
0.5
1.0
1.5
2.0
[S]

FIGURE 11: Lineweaver-Burk plot of GST (D) initial velocity v/s varying CDNB concentrations, with 3 mM GSH remaining constant, in the presence (closed circles) and absence (open circles) of the inhibitor, hydroquinone.

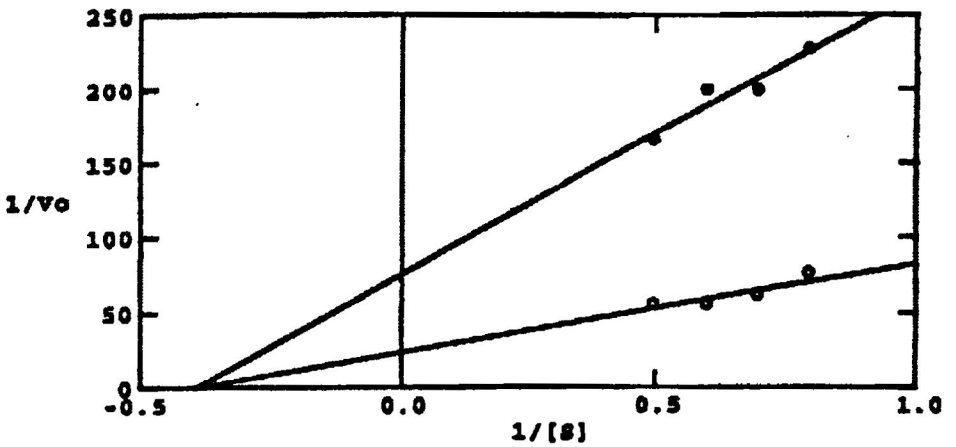

250
200
150
100
50
0
1/Vo
-0.5
0.0
0.5
1.0
1/[S]

FIGURE 12: Hyperbolic plot of GST (D) initial velocity v/s varying GSH concentrations, with 5 mM CDNB remaining constant, in the presence (closed circles) and absence (open circles) of the inhibitor, hydroquinone.

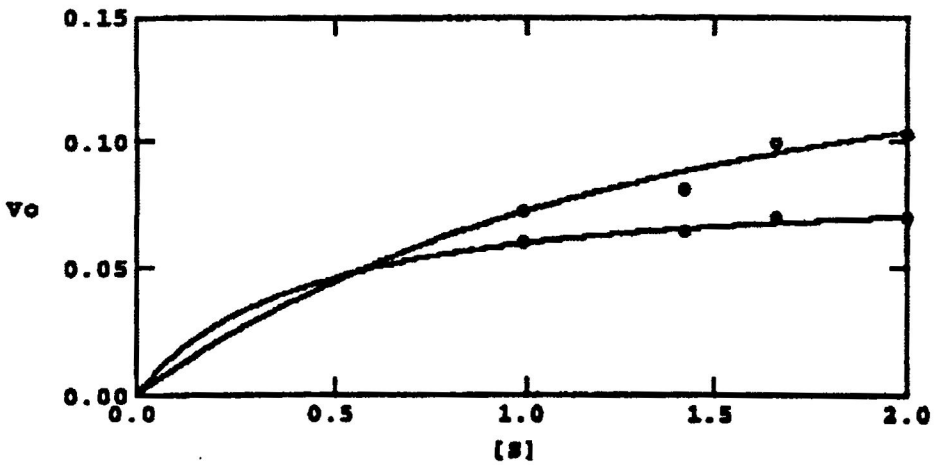

0.15
0.10
0.05
0.00
Vo
0.0
0.5
1.0
1.5
2.0
[S]

FIGURE 13: Lineweaver-Burk plot of GST (D) initial velocity v/s varying GSH concentrations, with 5 mM CDNB remaining constant, in the presence (open circles) and absence (closed circles) of the inhibitor, hydroquinone.

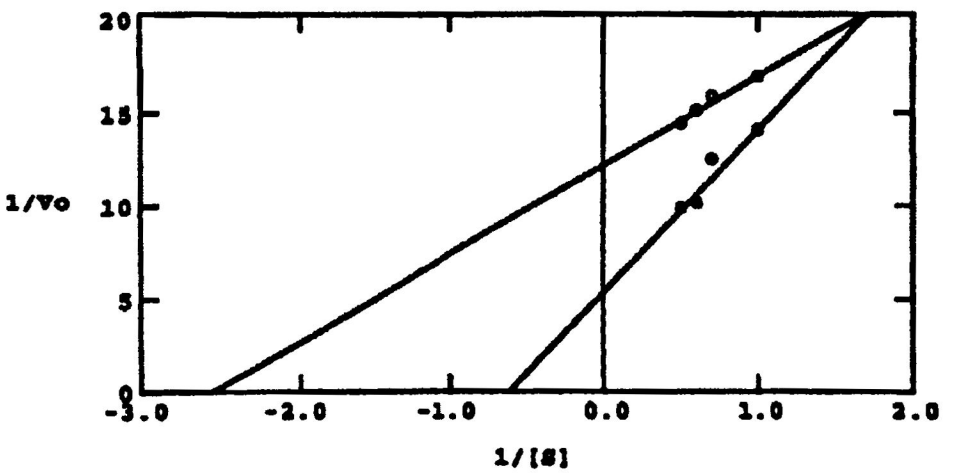

20
15
10
5
0
1/Vo
-3.0
-2.0
-1.0
0.0
1.0
2.0
1/[S]

TABLE 3: Glutathione s-transferase (high affinity form) kinetic parameters.

KINETIC PARAMETER	SUBSTRATE	+HYDROQUINONE	-HYDROQUINONE
K_m (mM)	GSH	0.391 ± 0.042	1.611 ± 0.548
V_m (nmol/min)	GSH	0.083 ± 0.002	0.188 ± 0.032
K_m (mM)	CDNB	2.514 ± 0.124	2.500 ± 0.003
V_m (nmol/min)	CDNB	0.013 ± 0.005	0.042 ± 0.005

In addition, the ratio of the Michaelis constants ($K_m^{CDNB}:K_m^{GSH}$) in the absence of hydroquinone does not significantly deviate from unity. These studies suggest, cumulatively, independent interactions between enzyme and substrate.

CHAPTER V

DISCUSSION

Chemoresistance is a primary concern regarding many diseases including antibiotic-resistant tuberculosis, sexually transmitted diseases and cancer treatment failure. During this phenomenon, tumors are rendered non-responsive to the therapeutic effects of anti-cancer agents. As such, many preneoplastic and neoplastic lesions progress to malignancy. Chemoresistance is complex and multiple mechanisms may be underlying this phenomenon. One form of chemoresistance is that of multi-drug resistance (Rodriguez et al., 1993; Ishikawa et al., 1993). In such a system, a 170 kilodalton phospho-glycoprotein (P-GP) acts as an active pump reducing the intracellular drug concentration (Rodriguez et al., 1993).

Another proposed mechanism of chemoresistance suggests that altered DNA topoisomerase activity results in enzymatic DNA cleavage in the presence of some antitumor agents. The antitumor agent does not block normal topoisomerase activity, but rather complexes with the topoisomerase subverting it in such a way as to render the enzyme a lethal instrument (Ross, 1985; Moscom and Cowan, 1988). Hence, the enzyme becomes a required co-factor for required drug action. The existing topoisomerase-drug complex has the capacity to cleave DNA molecules (Ross, 1985; Moscom and Cowan, 1988).

Induction of the GST genes have also been implicated in chemoresistance. As such, this gene and its product have been the topic of intense research in tumor cells. It has been shown that preneoplastic and neoplastic liver lesions display features different from those of normal liver cells in rats (Rao et al., 1992; Sato, 1989). These features include the loss of some normally present enzymes such as glucose 6-phosphatase, ATPase, and some phase I drug metabolizing enzymes. In addition, these features also include the increased activity of particular enzymes; γ-glutamyltranspeptidase, α-fetoprotein, glutathione s-transferase, and other phase II drug metabolizing enzymes. However, glutathione s-transferases have been reported as reliable markers for preneoplastic lesions and neoplastic tissues in the liver, as well as in other organs in rat and other species, including man (Sato, 1989; Rao, 1992).

This study has revealed the presence of multiple forms of GST, a high affinity and a low affinity form. The relevance of these cytosolic forms are still speculative. However, this phenomenon is not unique to human physiology as is seen in the redundance of the wobble code, overlapping hormonal activity, and the variety of immunoglobins comprising the humoral immune response. The presence of these forms of GST may result in a more versatile response and; perhaps, a more enhanced response to oxidative insults that may threaten the integrity of the cell. It is interesting to note that the

high affinity form is also the more active form and hence the more efficient form of GST but comprises less than half of the recovered forms of GST. In such a system, the more active form of GST would provide an immediate but brief response to oxidative insults whereas the less active form may provide a later but more sustained response to insults.

Multiple forms of GST may have another relevant role in hepatocytes. It is conceivable that, perhaps, one form may be more active under conditions that hinder the other. According to Lehninger (1975), the solubility of proteins can be influenced by pH. The minimal pH at which the protein remains soluble is referred to as that protein's isoelectric pH or isoelectric point (pI). At this point (pI) of minimal solubility one would also expect minimal activity. Hence, an enzyme would be least active at its isoelectric point. Considering fig. 6, the isoelectric point of the high affinity form is near neutral (pH 7). We also noted a sharp and steady decrease in activity at pH < 6.6 and pH > 7.3. This occurrence is probably due to protein denaturation. Jagt et al., (1985) isolated and characterized a high affinity form of GST, which they refer to as μ_4 having specific activity of 146±8 μmol/min/mg and pI of 6.19. These values are in reasonable agreement with our findings. They also report isolating a low affinity form of GST which they refer to as form III, with specific activity 62±1 μmol/min/mg with pI 8.72. Again, the activity of this form is in agreement with our findings.

Hence, it appears that the low affinity form is capable of activity under conditions which would compromise the activity of the high affinity form.

The electrophoretic behavior of both forms of GST yielded unique results. GST has been shown to be dimers of approximately 27 kd on an electrophoretic gel (Jagt et al., 1985). Jagt et al., have shown both forms of GST existing as a single band of approximately 27 kd on an electrophoretic gel. SDS-PAGE have resolved our GST samples as single bands comigrating with the 42 kd molecular weight marker. An additional protein, approximately 75 kd, co-purified with the high affinity form. Densitometric scans suggest that this protein comprises 15% of this sample (Area count = 2.55E-13 nm^2.; data not shown). Modification of the electrophoretic conditions; 1-5% SDS, 1-5% β-mercaptoethanol, 1-5% dithiotrietol, heated sample between 90°C-100°C for 5-45 minutes, did not alter the electrophoretic behavior of the isolated GST. The discrepancy in the molecular weight may be due to the source of GST. Jagt and his colleagues obtained normal human liver samples at autopsy from an adult female who died from accidental causes. Our samples were obtained from human hepatoma cells in culture. The proteins from these tumor cells may have been structurally altered resulting in abnormal structural stability. This occurrence would account for the seemingly monomeric behavior of GST.

Kinetic analyses were limited to the partially purified

high-affinity isoenzymes since this form (fraction D) was most active with the substrate CDNB. The apparent K_m for varying CDNB in the absence of the inhibitor, hydroquinone (HQ), was 2.50 mM (See Table 3 for kinetic parameters). The maximal rate of catalysis was 0.042 nmol/min. In the presence of HQ, the apparent K_m was unchanged, 2.51 mM, while V_{max} was 0.013 nmol/min. The intersection of both curves occur on the abscissa and is indicative of noncompetitive inhibition in the presence of hydroquinone. The possible enzyme forms present may be represented as E, EI or ESI, where EI is analogous to the enzyme-inhibitor complex and ESI is analogous to the enzyme-inhibitor-CDNB complex. These forms complexed to the inhibitor exhibited reduced activity. The unchanging K_m value indicated that the enzyme forms present (E & EI), which can combine with CDNB, have equal affinities for CDNB (Segel, 1975). The apparent K_m value, therefore, results from the distribution of available enzyme between the "full affinity" and "no affinity" forms (Segel, 1975).

The apparent K_m value for varying GSH was 1.61 mM while the maximal rate was 0.18 nmol/min in the absence of HQ. The inhibitor reduced the rate, 0.08 nmol/min., for product formation while markedly increasing the affinity, K_m= 0.039 mM, of the enzyme for the substrate. The plots (Fig. 13) intersect above the 1/[S] axis at a point greater than $1/V_{maxi}$ and a positive 1/[S] value unlike that of competitive or noncompetitive inhibition. The ESI/EI equilibria is

suggestive of a system displaying partial uncompetitive inhibition; however, the reciprocal plots are not parallel. This phenomenon is indicative of mixed-type inhibition (Segel, 1975).

The ratio of the Michaelis constants ($K_m^{CDNB}:K_m^{GSH}$) in the absence of hydroquinone does not significantly deviate from unity. This occurrence may suggest that the interaction between the primary substrate (S_1) and the unbound enzyme (E) does not significantly influence the interaction between the complex ($E\text{-}S_1$) and the secondary substrate. Hence, cooperativity appears to be negligible.

Cumulatively, these occurrences suggest a random interaction of GST with its substrates and possibly sequential binding of these substrates. The high affinity isoenzymes apparently possess multiple catalytic forms since random interaction with its substrates is preferential. This phenomenon may further be supported by the appearance of multiple ionic forms in which catalytic activity is maintained (Fig. 6). An effective therapeutic agent should be able to interact with these forms. In addition, aromaticity appears to be unimportant in terms of GST interaction, since negligible activity resulted with DCNB. This occurrence confirms the stereospecific nature of GST.

CHAPTER VI

CONCLUSIONS

Many chemotherapeutic agents are inactivated by glutathione s-transferase. Hence, these carcinostatic compounds may serve as potential substrates of GST and, as such, are rendered ineffective. The concept of drug overdose to compensate for potential drug detoxication is inefficient since many of these carcinostatic compounds induce deleterious side effects. The kinetic analysis of GST in human hepatoma cells may represent a convenient method of determining topical relationships/interactions with its substrates. As such, tumors may be sensitized to the effects of the chemoprotectant by minimizing GST activity.

This study has shown evidence of the random interaction of substrates with GST. Aromaticity appears to be unimportant in GST's activity. The presence of multiple forms of cytosolic GST may comprise an adaptive biotransformation system capable of responding to oxidative insults which may compromise the integrity of the cell. In addition, these enzymes appear to be more structurally stable in tumor hepatocytes than normal hepatocytes.

These findings may elucidate plausible mechanisms of drug-protein interaction and ultimately drug design. These studies, coupled with other electronic and stereospecific considerations, may be promising in enhancing chemotherapeutic efficacy.

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